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13. ABSTRACT *(Maximum 200 words)*

We developed new approaches with high resolution transmission electron microscopy in order to image beam-labile molecular structures which are important to the Navy in biofouling. In particular, we studied the adhesive components of two important marine biofoulers, Achnanthes and Stauronesis. We found interesting molecular structures which may contribute to the attachment of these diatoms to substrates such as ship and submarine surfaces. We produced a dichotomous key to help assess and describe in an objective way the complex molecular structures observed. We studied the effects of electron beam irradiation of the samples and learned that the uranyl acetate negative stain "protects" the samples from degradation during irradiation. These studies will augment x-ray crystallographic investigations in understanding the structural basis for unwanted molecular adhesion which is so common in the marine environment.

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FINAL REPORT

GRANT NUMBER: N000 14-95-1-0933

PRINCIPAL INVESTIGATOR: Dr. R. Malcolm Brown, Jr.

INSTITUTION: The University of Texas at Austin, Botany Department

GRANT TITLE: Atomic and Molecular Imaging of Adhesive Molecules

AWARD PERIOD: 1 May 1995 - 30 April 1998

OBJECTIVE: To investigate the structure of extracellular polymeric substance (=EPS) secreted by marine diatoms using low electron beam dose combined with high resolution transmission electron microscopy. To understand low dose preservation of beam-labile materials.
To collaborate with various investigators who are using multidisciplinary approach and to investigate their materials for better understanding and controlling unwanted mass of aquatic organism's adhesion.

APPROACH: Initially, we focused our investigation on the adhesive substances of two common marine diatoms involved in biofouling: Achnanthes and Stauronesis. We developed an in situ method for the generation of diatom adhesive material on TEM grids. We studied the pad structure of Achnanthes and the slime track structure of Stauronesis. We characterized the molecular structure of these materials using high resolution transmission electron microscopy and the preparation method of negative staining with uranyl acetate. Images were captured digitally and processed for maximum useful contrast and resolution. We developed a dichotomous key which aided in describing in an objective manner, the various molecular structures observed. Our later investigations have concentrated on determining how specimens can be so well preserved under electron irradiation conditions which seemingly would degrade the specimens.

ACCOMPLISHMENTS: Our first objective was to examine two different forms of the EPS synthesized in situ on the grid during 2 - 3 days in culture. (a) the pad and its radiating material, and (b) the insoluble slime secreted, probably during diatom locomotion. A very densely packed, spatially

distinct region of the pad where the stalk attaches to the substratum, shows radiating arms toward the periphery.

At the margin of these arms where the material is very thin, two molecular forms were observed and recorded. First, a linear, thread-like polymeric material which measured 1.2 nm in diameter, appears to be cross-linked. This arrangement may assist in stabilizing the radially elongated polymers as they are extruded from the diatom. The second form, a more globular structure, is located adjacent to the stalk. Because of the electron density of this region, it is difficult to discern the molecular structure. Bacteria frequently are embedded in the pad, and some of these bacteria themselves have highly organized fibrillar protrusions. The bacteria may help to form the so-called "conditioning" layer upon which the stalk pad material is attached. When Achnanthes was grown for short period of time (2.5 hr) the stalks were not produced, but instead an insoluble slime track material consisting of linear structures (about 3.0 nm in diameter) with a helical arrangement was observed. This material may also assist and promote the attachment of the diatom by its stalk.

The diatom, Stauroneis sp., grew much faster than Achnanthes and produced extracellular material as it glided on the surface to form a thin film composed of two distinct structural morphologies. The diameter of a fibrillar material is about 1.1 nm. The non-fibrillar structure, suggested by Dr. Weatherbee to be a glycoprotein, is approximately 10.0 nm in diameter and appears to have highly branched features.

We developed a catalog of diatom images and made a brief description for each representative series. Then we developed a dichotomous key for providing an initial objective assessment of the molecular structure. Because we were treading in unknown territory with regard to anticipation of what we might observe at the molecular level, we required an objective notation system to describe the molecular structures. An example key is included in the Appendix.

We applied Tinopal LPW, a brightener agent that causes disruption of crystallization process of glucan chains into the cellulose I allomorph (Acetobacter xylinum), to check if the progressive assembly of bioadhesive polymer clusters in the presence of the dye leads to an alteration of the adhesion. Unfortunately the agent precipitated with the sea water making the electron microscopic analysis impossible.

We collaborated with Dr. Michael Gretz of Michigan Technological University in Houghton on the attachment of diatoms to the substrate via the production of gelatinous stalks of Achnanthes longipes, and Dr. Valerie Vreeland of

the University of California of Berkeley on the roles of bromoperoxidase and polyphenols in formation of extracellular glues during initial adhesion by reproductive propagules of marine algae.

Dr. Gretz sent freeze-dried adhesive material of Achnanthes longipes for detailed high resolution analyses and for comparison with regular air dried specimen preparations, to test if air drying causes any artifact-induced distortion. The results of the freeze-dried material are presented in Figures 1-12. We observed various structural entities. Several elongated filamentous structures which measured 1.4 nm in diameter. We also observed globular material or clumps of these globular structures. Similar fibrillar and globular structures were also observed in our earlier analysis of air dried specimens of peripheral parts of the pad.

Dr. Vreeland sent purified bromoperoxidase from the brown alga Fucus and the red alga Corallina for high-resolution ultrastructural comparison of enzyme subunit organization. Unfortunately the process of purification (using SDS) created significant contamination making EM analysis very difficult.

In our latter studies, we investigated how the electron beam interacts with the specimens prepared under the conditions of negative staining with uranyl acetate (UA). We found that the UA crystallizes in the beam and surrounds the specimen thus protecting it from translational movement upon e-beam bombardment. Even though covalent bonds may have been broken by inelastic collisions, the overall primary and secondary structure remains intact. These discoveries provide a new basis for understanding the efficacy of this approach.

CONCLUSIONS: We have demonstrated that high resolution transmission electron microscopy of heretofore unknown polymeric materials involved in attachment of two well known biofouling diatoms can be imaged and characterized. The features of these materials can provide the molecular basis for understanding how biopolymers achieve such a strong, tenacious bond with the substrate. We also have a better understanding of the preservation of molecular structure under e-beam interactions.

SIGNIFICANCE: Through demonstration of the molecular structure of attachment biopolymers from two important marine biofoulers, it is possible to test other materials from different organisms which are involved in the biofouling process. The molecular dissection of the structure is important in developing models of contact adhesion and finding ways to disrupt such bonds. Conventional x-ray diffraction is capable of providing

molecular structural details; however, since a majority of the material cannot be crystallized for x-ray, the high resolution TEM approach offers a practical and useful alternative approach for characterization of biofouling agents at the molecular level.

PUBLICATIONS AND ABSTRACTS (for the total period of the grant):

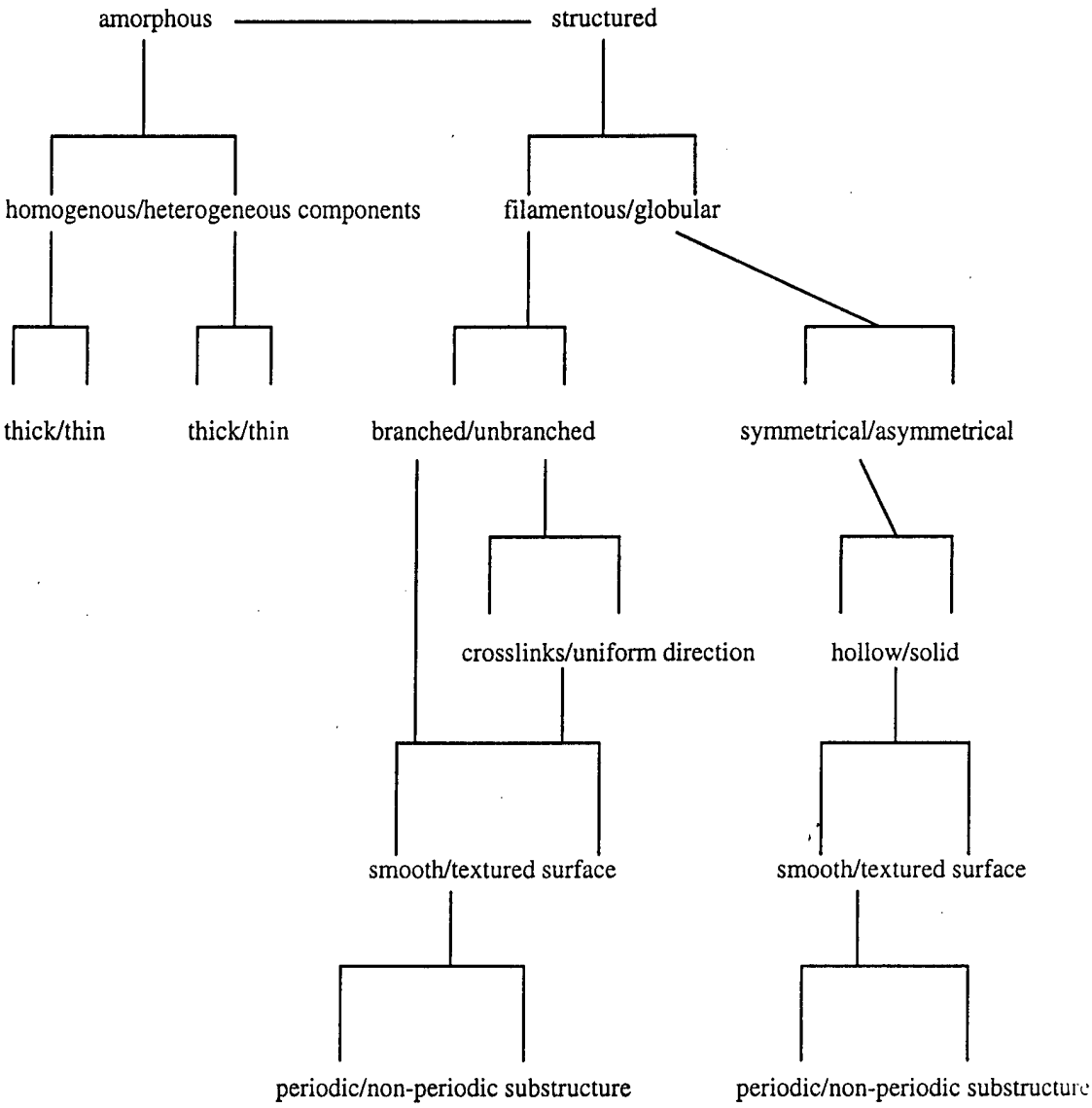
Brown, Jr. R. M., Saxena, I. M., and Kudlicka, K. (1966). Cellulose biosynthesis in higher plants. Trends in Plant Science 1:149-156.

Lee, H. J., and Brown, Jr., R. M. (1997). A comparative structural characterization of two cellobiohydrolases from Trichoderma reesei: a high resolution electron microscopy study. J. Biotechnology 57: 127-136.

In preparation:

Brown, Jr., R. M., Cofas, K.C., Sharp, J.A., and Spires, T. 1999) Molecular imaging of ornithine decarboxylase and bacitracin using low dose high resolution transmission electron microscopy.

Dichotomous Key for Describing Diatom Adhesion Molecules



Important Notations During the Observation of Diatoms

1. Is the diatom damaged?
2. Is the material coming from one or more points on the diatom's frustule?
3. Are there bacteria present?
4. What is the condition of the Formvar surrounding the diatom?